Research Article

Theme: Nanoparticles in Vaccine Delivery

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Induction of Death Receptor CD95 and Co-stimulatory Molecules CD80 and CD86 by Meningococcal Capsular Polysaccharide-Loaded Vaccine Nanoparticles

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ABSTRACT. *Neisseria meningitidis* is a leading cause of bacterial meningitis and sepsis, and its capsular polysaccharides (CPS) are a major virulence factor in meningococcal infections and form the basis for serogroup designation and protective vaccines. We formulated a novel nanovaccine containing meningococcal CPS as an antigen encapsulated in albumin-based nanoparticles (NPs) that does not require chemical conjugation to a protein carrier. These nanoparticles are taken up by antigen-presenting cells and act as antigen depot by slowly releasing the antigen. In this study, we determined the ability of CPS-loaded vaccine nanoparticles to induce co-stimulatory molecules, namely CD80, CD86, and CD95 that impact effective antigen presentation. Co-stimulatory molecule gene induction and surface expression on macrophages and dendritic cells pulsed with meningococcal CPS-loaded nanoparticles were investigated using gene array and flow cytometry methods. Meningococcal CPS-loaded NP significantly induced the surface protein expression of CD80 and CD86, markers of dendritic cell maturation, in human THP-1 macrophages and in murine dendritic cells DC2.4 in a dose-dependent manner. The massive upregulation was also observed at the gene expression. However, high dose of CPS-loaded NP, but not empty NP, induced the expression of death receptor CD95 (Fas) leading to reduced TNF- α release and reduction in cell viability. The data suggest that high expression of CD95 may lead to death of antigen-presenting cells and consequently suboptimal immune responses to vaccine. The CPS-loaded NP induces the expression of co-stimulatory molecules and acts as antigen depot and can spare antigen dose, highly desirable criteria for vaccine formulations.

KEY WORDS: CD80; CD86; CD95; co-stimulatory; nanoparticulate vaccine.

INTRODUCTION

Neisseria meningitidis (*N. meningitidis*) is primarily the single largest cause of meningitis and septicemia globally (1). Meningococcal infections cause rapidly fulminant sepsis leading to death of a previously healthy individual within hours. With the advancement of intensive care and treatment of the disease, the mortality rate has been considerably

reduced; nonetheless, the survivors often suffer from serious morbidity and tissue and neurological damage. Hence, prevention of meningitis through vaccination is desirable.

Meningococci have a polysaccharide capsule of which there are 12 distinct serogroups with A, B, C, W-135, and Y serogroups responsible for the majority of disease (2). Meningococcal capsular polysaccharides (CPS) are major virulence factor (2, 3) and form the basis of currently licensed vaccines Menactra™ (Sanofi Pasteur quadrivalent meningococcal conjugate MCV4 vaccine), Menomune® (Sanofi Pasteur unconjugated quadrivalent meningococcal polysaccharide MPSV4 vaccine), and Menveo® (Novartis quadrivalent conjugate meningococcal vaccine). Childhood vaccination has been shown to induce the herd immunity effect by reducing nasopharyngeal carriage (4–6). The current polysaccharide meningococcal vaccines that are available are very expensive. Meningococcal vaccines are poorly immunogenic in infants due to their underdeveloped immune responses. Since the incidence of meningococcal disease is highest in very young children under the age of 2, the FDA very recently approved the use of meningococcal conjugate vaccines Menactra™ and Menveo® in toddlers and infants as

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young as 9 months old (7). However, these conjugate vaccines require booster doses to maintain a protective immune response, continuous refrigeration, and administration by needle use, in addition to the high cost of chemical conjugation and limitation of producing and distributing conjugate vaccines which is inherent to all currently licensed vaccines. A novel nanotechnology-based meningococcal vaccine formulation can be a promising therapeutic against meningococcal infections (8).

We recently reported a novel vaccine formulation of meningococcal CPS incorporated in nanoparticles (9). We demonstrated that these vaccine-loaded nanoparticles are taken up by antigen-presenting cells and induced a robust innate immune response, a prerequisite for inducing adaptive immunity (9). The novel vaccine nanoparticle formulation has many advantages since it does not require chemical conjugation to a protein carrier, biodegradable, slowly release the antigen, highly immunogenic, inexpensive, easy to manufacture on large scale, highly stable, and does not require the cold chain in the logistics of vaccine delivery which reduce the cost and increase the availability (10–12). Furthermore, these vaccine nanoparticles designed for oral delivery *via* the mucosal route help induce an optimal immune response. Our group reported the efficacy of oral vaccine nanoparticles loaded with antigens such as *Salmonella* polysaccharides or *Mycobacterium tuberculosis* (12, 13).

Optimal antigen concentration in a vaccine formulation is imperative for an effective adaptive immune response. Antigens need to provide the co-stimulation while being expressed on the antigen-presenting cells (APCs) to induce an effective adaptive immune response. However, antigen concentration that induces the death pathway leading to the death of the T and B cells is not desirable (14, 15). Thus, the antigen dose is critical and has a narrow window between effectiveness and activation of death receptor; it is crucial that the antigen concentration is optimal for antigen presentation and co-stimulation of immune cells with minimal activation of the death pathway that lead to suboptimal immune response.

The co-stimulatory signals are required along with the antigen presentation *via* the major histocompatibility complex (MHC) complex on the APCs for its effective communication with T cells (16). CD80 and CD86 are well-studied co-stimulatory molecules expressed on APCs and markers of dendritic cell maturation (17). CD80 and CD86 bind to their ligand, CD28, on the T cell and provide secondary signal for antigen presentation. For an ideal vaccine antigen, once taken up by the APCs, they should provide ample expression of these co-stimulatory molecules in order to establish T cell contact and further provide T cell-mediated immunity (18–20).

CD95 (also called Fas, APO-1, or TNFRSF6) is a membrane protein that belongs to the TNFR family and is commonly called as death receptor. Binding of its physiological ligand, CD95L, to CD95 causes the activation of the death pathway (14). Upon ligation of CD95 with its ligand CD95L, it activates the death signaling pathway leading to formation of the death-inducing signal complex (DISC), and it is orchestrated by the action of a set of proteases in the cell, called caspases (15). Depending on the cell type, there are two mechanisms, intrinsic

and extrinsic pathways, which ultimately lead to programmed cell death, apoptosis (14).

It has been shown that some bacterial capsular polysaccharides, such as the *Cryptococcus neoformans*, glucuronoxylomannan (GXM), induce the upregulation of CD95L on the macrophages and, as a consequence, apoptosis of the lymphocytes (14, 21, 22). This same phenomenon is reported in human protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) (23). When vaccinated with a recombinant adenovirus expressing an immunodominant parasite antigen, there was induction of apoptosis by CD95L which was overexpressed and led to suboptimal CD8+ T cell-mediated immune response which lead to failure of the vaccine (23–26). However, it is not known whether *N. meningitidis* CPS induces CD95 overexpression at high dose leading to apoptosis of immune cells such as the dendritic cells and other antigen-presenting cells resulting in a suboptimal immune response.

In this study, we investigated antigen presentation by murine dendritic cells (DC) and by human macrophages pulsed with meningococcal CPS-loaded nanoparticles. Our finding shows that these meningococcal CPS-loaded nanoparticles induced the expression of the co-stimulatory molecules, CD80 and CD86. However, a high dose of CPS antigen induced the expression of the death receptor CD95 in murine DC as well as in human and murine macrophages leading to cell death. Our data reveal an unknown pathophysiological role for meningococcal CPS in inducing CD95 death receptor. Our data also suggest that lower CPS dose loaded in vaccine nanoparticle would spare antigen and reduce the death of antigen-presenting cells.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium, Dulbecco's Modified Eagle medium, fetal bovine serum (FBS), penicillin/streptomycin, sodium pyruvate, and nonessential amino acids were obtained from Cellgro Mediatech (Herndon, VA). RAW264.7 and THP-1 cell lines were purchased from ATCC (Manassas, VA). Dendritic cells (DC2.4) were given as a kind gift from Dr. Kenneth L. Rock (Dana-Farber Cancer Institute, Inc., Boston, MA, USA). ELISA kits for cytokine measurements were purchased from R&D systems (Minneapolis, MN). The vaccine-grade polysaccharide antigens were a kind gift received from Dr. Seshu Gudlavalleti (JN Medical Corporation, Omaha, NE, USA). Sterile and endotoxin-free bovine serum albumin (BSA) used to formulate the protein-based nanoparticles was purchased from Sigma-Aldrich. Antibodies used to stain human and murine CD80, CD95, and CD86 for flow cytometric analysis were purchased from eBioscience laboratories (San Diego, CA).

Preparation of Nanoparticles Using BSA

The biodegradable nanoparticles were prepared by the previously established method by our laboratory using the Buchi Mini Spray Dryer B-191 (9, 10, 13, 27). Briefly, a 1% solution of sterile BSA in sterile water was prepared and kept

for overnight crosslinking with glutaraldehyde. Excess glutaraldehyde was neutralized with sodium bisulphate the following day. Meningococcal polysaccharide antigen from serogroup A, CPS-A, was added to the solution and spray dried through a 0.5-mm nozzle at a flow rate of 20 mL/h to obtain the nanoparticles. Particle characterization, polysaccharide content, and recovery yield were calculated as previously described (9).

The meningococcal serogroup A CPS (NMA) is the major disease-causing serotype in the meningitis belt area in the African continent (3). Vaccine delivery efforts have been impeded by the unavailability of the cold chain required to preserve the currently used conjugate meningococcal vaccines. Therefore, for proof-of-concept to provide a nanotechnology-based vaccine formulation that does not require refrigeration, we used NMA as vaccine antigen.

Cell Culture

Murine DC 2.4 and human macrophage-like monocyte THP-1 cells were grown in RPMI 1640 with L-glutamate supplemented with 10% FBS, 50 IU/mL of penicillin, 50 µg/mL of streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. Culture flasks were incubated at 37°C with humidity under 5% CO₂. Murine RAW264 macrophages were grown in Dulbecco's Eagle medium supplemented with incubated as mentioned above.

Gene Expression Methods

Some 5×10^6 THP-1 cell/mL was transferred to six-well formats and then stimulated with 10 µg/mL of meningococcal CPS derived from the endotoxin-free meningococcal serogroup B *lpxA* mutant as described (2). Unstimulated cells were used as controls for basal gene expression level. Cells were incubated overnight at 37°C under 5% CO₂. RNA was isolated using RNeasy Mini kits (Qiagen) as previously described (2). The experimental cocktail for real-time PCR was prepared in a sterile boat as follows: 1,275 µL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 102 µL of diluted cDNA, and 1,173 µL of ddH₂O. Real-time PCR was then performed using RT² profilerTM PCR Array (SuperArray Bioscience Corporation) in 96-well format pre-loaded with the primers. Human toll-like receptor signaling pathway and human apoptosis pathway RT² profilerTM PCR Array profile the expression of 84 genes related to TLR-mediated signal transduction and apoptosis pathways. In addition to primers, the array contains all positive and negative control required for real-time PCR procedure. To start the real-time PCR reaction, 25 µL of experimental cocktail mix was carefully added to each well in the RT² PCR Array using multi-channel pipette then was tightly sealed with the optical adhesive film. The PCR parameters were set as follows: 2 min at 50°C, 10 min at 95°C, and 45 cycles of 95°C for 15 s followed by 1 min at 62°C. For data analysis, the Excel-based PCR Array data analysis template (downloaded from this link: <http://www.superarray.com/pcrarraydataanalysis.php>) was used. Gene expression profiles were automatically calculated from threshold cycle data generated from the real-time instrument, and any C_t value equal or greater than 35 will be considered negative as described (2).

Quantification of Co-stimulatory Molecule (CD80 and CD86) Expression

CD80 and CD86 expression was checked by plating 5×10^4 dendritic cells (DC2.4) per well in a 96-well plate and incubated at 37°C for 24 h for them to adhere and stabilize. The cells were pulsed with varying concentrations of CPS loaded into nanoparticles (1, 2, 4, and 8 µg/mL) and incubated at 37°C for 16 h. CPS solution in the same concentration and equal amount blank nanoparticles were used as controls. The amount of bioactive NMA antigen encapsulated in the vaccine nanoparticles was calculated as described (9). The cells were then washed to remove the particles and cell samples were obtained which were treated with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled CD80 and CD86 marker (eBioscience laboratories, San Diego, CA) for 1 h at 4°C. The cells were washed and sample measurements were then acquired on BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA). The same method was used to assess CD80 and CD86 expression in THP 1 cells pulsed with CPS-loaded nanoparticles.

CD95 Expression in DC2.4 and THP-1 Cells

The expression of CD95 was examined by plating 5×10^4 dendritic cells (DC2.4) per well in a 96-well plate and incubated at 37°C for 24 h to adhere and stabilize. Adherent cells were pulsed with 5 and 10 µg of CPS-loaded nanoparticles and further incubated at 37°C for 16 h. Similar concentration of CPS in solution and equal amount of blank nanoparticles were used as controls. The pulsed cells were then washed to remove excess nanoparticles that were not taken up by dendritic cells and stained for CD95 marker. Briefly, the harvested cells were treated with FITC-labeled CD95 marker (eBioscience laboratories, San Diego, CA) for 1 h at 4 °C. The cells were washed and sample measurements were then acquired on BD Accuri C6 flow cytometer. Similarly, the expression of CD95 in THP 1 cells pulsed with CPS-loaded nanoparticles was assessed as above.

Cytokine Measurement from THP-1 Cells

The human cytokine TNF-α released from THP-1 cells stimulated with nanoparticles containing meningococcal capsular polysaccharide antigens was quantified by DuoSet ELISA (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. Supernatants were diluted in reagent diluents (0.1% BSA and 0.05% Tween 20 in Tris-buffered saline (28).

Nitric Oxide Release from DC2.4 and RAW264 Cells

Freshly grown adherent DC2.4 cells and RAW264 macrophages were harvested, washed and resuspended in Dulbecco's complete media, counted, and adjusted to 10^6 cell/mL. Aliquots (250 µL) were then dispensed into each well of a 96-well plate at final 250×10^3 cell density prior to stimulation with either nanoparticle doses containing meningococcal CPS, blank nanoparticles, and CPS solution. The induced cells were incubated overnight at 37°C with 5% CO₂ and supernatants were harvested and saved. Nitric oxide

release was quantified using the Greiss chemical method as previously described. Briefly, the Griess chemical method was used to detect nitrite (NO_2) accumulated in supernatants of induced RAW264 macrophages. Griess reagent was freshly prepared by mixing equal volumes of 1% sulfanilamide and 0.1% *N*-(1-naphthylethylenediamine) solutions. One hundred microliters of cell supernatants was transferred into a 96-well plate to which 100 μL of Griess reagent was added. The plate was mixed gently, incubated for 10 min at room temperature, and read at 540 nm using a microplate reader (EL312e; BIO-TEK Instruments, Winooski, VT). The optical densities were correlated to the concentration of nitrite. Nitrite was quantitated using the standard curve of sodium nitrite (1 mM stock concentration in distilled water further diluted to the highest standard at 100 μM followed by serial dilutions to 1.56 μM) (28).

Cell Viability Measurements

The vital dye trypan blue (0.4%) stored in a dark bottle was used for measuring cellular viability as described before (9). Equal volumes of 0.4% trypan blue and cell suspension were mixed and allowed to stain for 3 min prior to quantification of viable cells.

Statistical Analysis

All experiments were performed in quadruplets. Mean values $\pm$ SD and *P* value (Student's *t* test unpaired, two-tailed distribution) were determined individually for all experiments with Microsoft Excel software. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Meningococcal CPS as Vaccine Antigen-Induced Robust Induction of Co-stimulatory Molecules Required for Effective Antigen Presentation

Effective antigen presentation is essential for optimal adaptive immune response. We examined whether meningococcal CPS as a vaccine antigen can induce the expression of multiple co-stimulatory molecules that impact optimal antigen presentation. Gene expression of various co-stimulatory molecules in THP-1 macrophages induced with 10 $\mu\text{g}/\text{mL}$ of meningococcal CPS showed highly significant upregulation of dendritic cell maturation markers CD80 and CD86 at 2,184- and 30-fold, respectively. The upregulation of other co-stimulatory molecules that impact antigen presentation and T cell activation was also highly significant including CD40 (17-fold), CD40L (9-fold), and CD137 (also called TNFRSF9 or 4-1BB) (115-fold). In addition, the death-inducing receptor CD95 (Fas receptor) and its ligand CD95L were significantly upregulated with an 18- and 62-fold increase, respectively. The data suggest that meningococcal CPS as a vaccine antigen can induce the gene expression of co-stimulatory molecules required for effective antigen presentation.

To examine whether meningococcal CPS as vaccine antigen encapsulated in nanoparticles would retain the ability to induce the expression of co-stimulatory molecules CD80 and CD86 on the surface of APCs, we employed human THP-1 macrophages

and murine dendritic cells DC2 pulsed with meningococcal CPS-loaded nanoparticles or stimulated with CPS in solution. We observed a significant increase in the surface expression of CD80 and CD86 in a dose-dependent manner when THP-1 cells were pulsed with CPS-loaded nanoparticles but not with empty blank particles (Fig. 1). Noteworthy is that meningococcal CPS in solution, at equal concentration to that of CPS tethered in nanoparticles, induced a much lower expression of CD80 and CD86 in THP-1 cells. Similarly, the surface expression of murine CD80 and CD86 on dendritic cells DC2.6 pulsed with meningococcal CPS-loaded nanoparticles was dose-dependent (Fig. 2). The data suggest that encapsulation of CPS as vaccine antigen in nanoparticles enhanced the expression of co-stimulatory molecules due to slow release of antigen and enhanced uptake by macrophages as we previously reported (9).

Surface Expression of CD95 on Antigen-Presenting Cells Pulsed with Meningococcal CPS-Loaded Nanoparticles

Meningococcal CPS as vaccine antigen induced the gene expression of the death-inducing receptor CD95 and its ligand CD95L (Fas/FasL). To confirm that meningococcal CPS encapsulated in nanoparticles can induce the surface expression of CD95 on antigen-presenting cells, we pulsed

Fig. 1. Dose-dependent expression of CD80 and CD86 molecules in THP-1 cells pulsed with meningococcal vaccine-loaded nanoparticles. THP-1 cells (5×10^4) were pulsed with increasing doses of nanoparticles loaded with meningococcal CPS serogroup A (CPS NP), empty nanoparticles (blank NP), or CPS in solution for 24 h. The expression of co-stimulatory molecules CD80 (a) and CD86 (b) was detected using flow cytometer following staining with FITC- and PE-labeled CD80 and CD86 marker, respectively. Error bars represent the standard deviation from the mean of four independent experiments. *P* values were calculated using unpaired Student's *t* test and two-tailed distribution comparing CPS NP to the blank NP and CPS solution. **P* < 0.05 and ***P* < 0.01 were significant

Fig. 2. Expression of co-stimulatory molecules CD80 and CD86 on dendritic cells. Murine DC2.6 cells (5×10^4) pulsed with increasing doses of nanoparticles loaded with meningococcal CPS serogroup A (CPS NP), empty nanoparticles (blank NP), or CPS in solution for 24 h. The expression of co-stimulatory molecules CD80 (a) and CD86 (b) was detected using flow cytometer following staining with FITC- and PE-labeled CD80 and CD86 marker, respectively. Error bars represent the standard deviation from the mean of four independent experiments. *P* values were calculated using unpaired Student's *t* test and two-tailed distribution comparing CPS NP to the blank NP and CPS solution. **P* < 0.05 and ***P* < 0.01 were significant

human THP-1 cells and murine dendritic cells with meningococcal CPS-loaded nanoparticles. The expression of CD95 was increased as the concentration of CPS was increased from 5 to 10 µg in both the THP-1 and DC2.6 cells (Fig. 3). Further, meningococcal CPS encapsulated in nanoparticles induced significantly higher expression of CD95 when compared to CPS solution or blank nanoparticles (Fig. 3). The data suggest that high expression of CD95 may lead to death of antigen-presenting cells and consequently suboptimal immune responses to vaccine. This is the first report to show that meningococcal CPS can induce CD95 (Fas) which implicate a pathophysiological role of meningococcal CPS during infection and vaccination.

Viability and Functionality of Antigen-Presenting Cells Pulsed with Meningococcal CPS-Loaded Nanoparticles

In order to determine the impact of CD95 induction on antigen-presenting cells, we pulsed human THP-1 cell, murine RAW264 macrophages, and murine dendritic cells DC2.4 with different doses of meningococcal CPS-loaded nanoparticles. We assessed APC viability using trypan blue exclusion and functionality as intact innate immune response, *i.e.*, ability to release TNF- α . A dose-dependent increase in the

Fig. 3. Induction of CD95 on antigen-presenting cells pulsed with meningococcal vaccine loaded nanoparticles. Human THP-1 cells (a) and murine DC2.6 cells (b) (5×10^4) were pulsed with nanoparticles loaded with meningococcal CPS serogroup A (CPS NP), empty nanoparticles (blank NP), or CPS in solution for 24 h. The expression of human (a) and murine (b) CD95 was detected using flow cytometer following staining with FITC-labeled CD95 marker. Error bars represent the standard deviation from the mean of four independent experiments. *P* values were calculated using unpaired Student's *t* test and two-tailed distribution comparing CPS NP to the blank NP and CPS solution. **P* < 0.05 and ***P* < 0.01 were significant

release of TNF- α from THP-1 cells incubated with CPS-loaded nanoparticles at the $1 \times$ concentration was observed reaching a high of about 1,000 pg/mL at CPS NP concentration of 2,560 ng/mL (Fig. 4). In contrast, THP-1 cells that were incubated with $10 \times$ concentration of CPS-loaded nanoparticles showed an initial increase of TNF- α released with increasing concentration of nanoparticles, but beyond a nanoparticle concentration of 6.4 µg/mL, the TNF- α release was decreased and eventually was completely inhibited at high dose of 25.6 µg/mL (Fig. 4). The empty nanoparticles on the other hand stimulated no release of TNF- α at any of the concentrations tested and did not reduce cellular viability even when used at high dose as previously reported (9). Further, reduced cell viability was observed at high dose of CPS-loaded nanoparticles (data not shown) suggesting that lack of innate immune response is due to cell death. Not only do these results reiterate the fact that CPS-loaded nanoparticles can induce cytokine release from human macrophage but they also demonstrate that high antigen dose can induce CD95 as shown above and modulate the innate immune response elicited. These results suggested that a higher antigen concentration could lead to reduced functionality of APCs, thereby leading to a suboptimal immune response.

Fig. 4. Meningococcal vaccine-loaded nanoparticles induced human TNF- α release. THP-1 cells (1 million cell/mL) were pulsed with increasing doses of nanoparticles loaded with meningococcal CPS serogroup A (NMA particles) at 1 $\times$ or 10 $\times$ concentration or with empty nanoparticles (blank NP) for 24 h. Human TNF- α release was quantified in the supernatants using the ELISA method. Error bars represent the standard deviation from the mean of three independent experiments. *** P <0.001 values of the group which received 10 $\times$ the concentration of CPS-loaded particles (NMA) when compared to the blank particles and NMA particles. There was a significantly low release of TNF- α from NMA particles as compared to 10 $\times$ concentration of particles (^{aa}P <0.01)

Similarly, innate immune recognition of meningococcal CPS-loaded nanoparticles in the murine RAW264 macrophages and in dendritic DC2.4 cells was examined. Murine RAW264 macrophages recognized CPS-loaded nanoparticles, but not the empty nanoparticles, and released nitric oxide in a dose-dependent manner (Fig. 5). The release of nitric oxide was observed with increasing concentrations of nanoparticles to ten times, and the release of nitric oxide increased proportionately. There is a significant difference between the release of nitric oxide by CPS-loaded nanoparticles at 1 $\times$ concentration and 10 $\times$ concentration. High dose of CPS-loaded nanoparticles leads to reduction in nitric oxide release suggesting an impaired responses due to cell death (data not shown). In contrast, the empty nanoparticles did not stimulate nitric oxide release from murine macrophages indicating that the nanoparticle matrix was inert and did not contribute to the innate immune response.

A similar pattern of nitric oxide release was observed in dendritic DC2.6 cells pulsed with different doses of meningococcal CPS-loaded nanoparticles (Fig. 6). Nitric oxide release was inhibited at high doses of meningococcal CPS-loaded nanoparticles again suggesting impaired functionality due to cell death. The cell viability was examined and showed that the as the concentration of CPS-loaded nanoparticles was increased, there was cell death. As the concentration increases from 1 to 10 μ g/well, the cell viability falls almost 40% (Fig. 6). Taken together, the data suggest that a high dose of meningococcal CPS-loaded nanoparticles induces CD95 which leads to APC death, consequently reducing innate immune responses and insufficient antigen presentation.

DISCUSSION

Upon vaccine administration, effective antigen presentation is a prerequisite for optimal adaptive immune responses.

Fig. 5. Dose-dependent nitric oxide releases RAW264 macrophages. One million cells/mL were pulsed with increasing doses of nanoparticles loaded with meningococcal CPS serogroup A (NMA particles) at 1 $\times$ or 10 $\times$ concentration or with empty nanoparticles (blank NP) for 24 h. Nitric oxide release in the supernatants was quantified by the Griess method. Error bars represent the standard deviation from the mean of three independent wells. This is a representative of four independent experiments. *** P <0.001 values of the group which received 10 $\times$ the concentration of CPS-loaded particles (NMA) when compared to the blank particles and NMA particles. There was a significantly low release of nitric oxide from NMA particles as compared to 10 $\times$ concentration of particles (^{aa}P <0.01)

The nature of vaccine antigen dictates its immunogenicity which is impacted by its uptake, processing, and presentation on the surface of APCs. Macrophages and dendritic cells are the major APCs that play an indispensable role in inducing adaptive immunity by activating T and B lymphocytes (29). Bacterial capsular polysaccharides are a major virulence factor and form the basis of vaccines. In contrast to protein antigens, CPS, a carbohydrate-linked polymer, does not induce T cell-dependent responses leading to suboptimal adaptive immunity. Therefore, the conjugation of CPS to a protein carrier directly impacts the immunogenicity of CPS polymers by inducing T cell-dependent immune responses

Fig. 6. Dose-dependent nitric oxide release from dendritic cells. Dendritic DC2.6 cells were pulsed with increasing doses of meningococcal CPS-loaded nanoparticles or CPS in solution and incubated for 16 h. Nitric oxide release was quantified in the supernatants using the Griess method. The red line, read on secondary axis on right, shows the cell viability at varying concentrations of CPS NP after 16 h of incubation. Reduction in the live cell count is seen as the dose of CPS is increased. Error bars represent the standard deviation from the mean of three independent wells. This is a representative of four independent experiments. Increased release of nitric oxide at high levels of CPS NP as compared to blank NP and CPS solution (*** P <0.001)

(30). In this study, we demonstrate that the novel meningococcal vaccine formulation that encapsulates CPS antigen in biodegradable nanoparticles can induce co-stimulatory molecules required for effective presentation. The novel formulation does not require conjugation to a protein carrier since the biodegradable nanoparticles are albumin-based and bind to the negatively charged meningococcal CPS polymers, thus act as antigen depot (31–33). We recently reported the uptake of these vaccine-loaded nanoparticles by macrophages as the main APCs and documented the slow antigen release leading to more robust innate immune responses (9). Here, we further characterize the ability of these novel meningococcal vaccine-loaded nanoparticles to induce effective antigen presentation in macrophages and dendritic cells. Our data show that vaccine-loaded nanoparticles induced CD80, CD86, and other co-stimulatory molecules with lower doses of antigen, thereby sparing antigen which is a great advantage for vaccine formulation. The expression of co-stimulatory molecules was observed at the gene level and further confirmed the protein expression on the surface of APCs, *i.e.*, macrophages and dendritic cells.

CD80 and CD86, well-studied co-stimulatory molecules, are expressed on APCs, upregulated upon cell activation, and bind to CD28 on the T cell, delivering a crucial signal for T cell activation together with the T cell receptor, subset differentiation, and effector function (34, 35). It is also known that CD80 and CD86 co-stimulation is required for a high avidity neutralizing antibody response; thus, we measured the expression of CD80 and CD86 in dendritic cells (20). This overexpression of CD80 and CD86 ensures the increase in the magnitude of both primary as well as secondary T cell response, ideal for a meningitis vaccine (36–38).

Activation of naïve T cells by APCs such as DC expressing MHC/peptide complexes and CD28 ligands (CD80 and CD86) leads to intense proliferation and clonal expansion. Memory T cells generated after a primary response expand rapidly upon re-encountering antigen and CD28 co-stimulation. When memory cells re-encounter antigen without sufficient CD28 co-stimulation, they fail to fully expand and clear pathogens (16, 37). Conversely, if naïve T cells are activated by APCs in the absence of CD28 co-stimulation, there is limited clonal expansion or even anergy. The resulting memory population is normal in terms of quantity but quality is affected and these memory cells do not re-expand optimally upon antigen re-encounter (18, 19). Thus, post-antigen exposure, the expression of CD80 and CD86 by the APCs, is vital for the robustness of the vaccine therapy. To examine the immunogenicity of the novel meningococcal vaccine-loaded nanoparticles, an *in vivo* animal vaccination study is under current investigation.

In this study, we report for the first time that meningococcal CPS can induce the expression of death receptor CD95 (Fas) at the gene level as well as on the surface of macrophages and dendritic cells. The induction of CD95 was dose-dependent especially at higher concentrations of CPS alone or when encapsulated in nanoparticles. However, CD95 overexpression has been attributed to cell death when it binds to its ligand CD95L (FasL) (14). In this study, both CD95 and CD95L were induced by meningococcal CPS. Indeed, high level of CD95 expression induced by high dose of meningococcal CPS antigen led to decreased macrophage and dendritic cell viability and inhibition of TNF- α and nitric oxide release as markers of innate immune responses in

APCs. It was evident that THP-1 cells were unable to release TNF- α after incubation with high antigen concentrations as the higher concentrations caused overexpression of CD95 and subsequent cell death. Previous report by Zughaier demonstrated that meningococcal CPS induced TLR2 and TLR4-MD-2 mediated inflammatory responses in macrophages (2). The induction of CD95 and CD95L by meningococcal CPS eludes to a novel pathophysiological mechanism that may contribute to the massive fulminant sepsis during meningococcal infection. Macrophage-induced apoptosis is emerging as a macrophage effector function; as these activated macrophages express CD95L, they are able to induce apoptosis of T cells (39).

CONCLUSION

Encapsulation of meningococcal CPS, as a vaccine antigen, in biodegradable nanoparticles induces the expression of co-stimulatory molecules necessary for effective antigen presentation by APCs like macrophages and dendritic cells. The vaccine-loaded nanoparticles act as antigen depot and can spare antigen dose, highly desirable criteria for vaccine formulations.

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Conflict of interest The authors have no conflict of interest.

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